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Cerebral Acetylcholine and Choline Contents and Turnover Following Low-Dose Acetylcholinesterase Inhibitor Treatment in Rats

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14. ABSTRACT

Rats were treated for three weeks with regular drinking water plus subcutaneous (sc) saline (0.5 ml/kg) injections three times/week; pyridostigmine bromide (PB) in drinking water (80 mg/L) plus sc saline injections three times/week; regular drinking water plus sc sarin (0.5 x LD_{50}) injections three times/week; or PB in drinking water plus sc sarin injections three times/week. Repeated doses of sarin, with or without PB, were devoid of acute toxicity during the three-week treatment period. Two, 4, and 16 weeks post-treatment, animals were given an intravenous pulse injection of choline labeled with 4 deuterium atoms (D4Ch) followed, 1 min later, by microwave fixation of the brain *in vivo*. Tissue levels of endogenous acetylcholine (D0ACh), endogenous choline (D0Ch), D4Ch, and ACh synthesized from D4Ch (D4ACh) were measured by GC-MS in hippocampus, infundibulum, mesencephalon, neocortex, piriform cortex, and striatum. Ch uptake from blood and ACh turnover were estimated from D4Ch and D4ACh concentrations in brain tissue, respectively. Statistically significant differences among brain regions were found for D0Ch, D4Ch, D0ACh and D4ACh at 2, 4 and 16 weeks post-treatment. Differences in these parameters between control and drug treatments were found only for D0ACh and D0Ch at 2 and 4 weeks. The results from these experiments do not support a delayed or persistent alteration in cholinergic function after exposure to low doses of PB and/or sarin.

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ABSTRACT

Male Sprague-Dawley rats were treated for three weeks with 1) regular tap drinking water plus subcutaneous (s.c.) saline (0.5 ml/kg) injections three times/week, 2) pyridostigmine bromide (PB) in drinking water (80 mg/L) plus s.c. saline injections three times/week, 3) regular tap drinking water plus s.c. sarin (0.5 x LD_{50}) injections three times/week, or 4) PB in drinking water plus s.c. sarin injections three times/week. Repeated doses of sarin, in the presence or absence of PB, were devoid of acute toxicity during the three-week treatment period. Two, 4, and 16 weeks post-treatment, animals were given an intravenous pulse injection of choline labeled with 4 deuterium atoms (D4Ch) followed, after 1 minute, by microwave fixation of the brain *in vivo*. Tissue levels of endogenous acetylcholine (D0ACh), endogenous choline (D0Ch), D4Ch, and ACh synthesized from D4Ch (D4ACh) were measured by gas-chromatography mass-spectrometry in hippocampus, infundibulum, mesencephalon, neocortex, piriform cortex, and striatum. Ch uptake from blood and ACh turnover were estimated from D4Ch and D4ACh concentrations in brain tissue, respectively. Statistically significant differences among brain regions were found for D0Ch, D4Ch, D0ACh and D4ACh at 2, 4 and 16 weeks post-treatment. However, differences in the values of these parameters between control and drug treatments were found only for D0ACh and D0Ch at 2 and 4 weeks, but not at 16 weeks post-treatment. In conclusion, the results from these experiments do not support a delayed or persistent alteration in cholinergic function after exposure to low doses of PB and/or sarin.

INTRODUCTION

The effects of acetylcholinesterase (AChE) inhibitors on the central nervous system (CNS) depend on their ability to cross the blood-brain barrier. Organophosphorus (OP) nerve agents can readily access the CNS and produce rapid lethal consequences. The acute toxic effects of exposure to the OP nerve agents, such as sarin and soman, stem primarily from the inhibition of AChE, causing an excessive accumulation of the neurotransmitter acetylcholine (ACh) resulting in persistent action on the cholinergic synapses. The acute effects of toxic doses of nerve agents on the levels of ACh and its precursor and degradation product choline (Ch) are well characterized in the brains of animals (Flynn and Wecker 1986; Fosbraey et al. 1990; McDonough, Jr. and Shih 1997; Shih and McDonough, Jr. 1997; Shih 1982). However, much less information is available on the persistent or delayed effects of exposure to low levels of these agents on ACh and Ch metabolism.

Toxicity of low levels of nerve agents has recently received attention due to reports of a conglomerate of symptoms, including cognitive alterations, balance disturbances, vertigo, and muscle aches and weakness (Haley et al. 1997), known as Gulf War Illness, in veterans of the 1991 Persian Gulf War (PGW). Exposure of PGW veterans to sub-symptomatic levels of sarin has been documented by field studies and modeling analyses of environmental contamination by this nerve agent (General Accounting Office 2003; McCauley et al. 2001), as well as epidemiological studies (Wolfe et al. 1998). Prophylactic administration of PGW service members with the carbamate AChE inhibitor pyridostigmine bromide (PB) as a preventive measure against nerve agents occurred during the PGW (Keeler et al. 1991). PB is a peripherally acting AChE inhibitor (i.e., does not cross the blood-brain barrier) that protects soldiers from the lethal effects of OP AChE inhibitors when given in anticipation of exposure to toxic nerve agents. The mechanism of this protection seems to be the pre-occupation of peripheral AChE reactive sites by PB, which become unavailable to the OP nerve agent, with subsequent restoration of enzyme activity due to spontaneous decarbamylation of AChE (Dirnhuber et al. 1979; Keeler et al. 1991; Kluwe et al. 1987; Koplovitz et al. 1992; Leadbeater et al. 1985). Based on above mentioned facts, Gulf War Illness has been ascribed by some clinicians, among other possible factors, to intake of PB and/or exposure to low levels of sarin (Haley 2001).

Our previous animal experimentation with the same low-level sarin and PB treatment regimen as in the present report has indicated effects on blood AChE activity during and shortly after treatment and delayed effects, 2 to 16 weeks post-treatment, on exploratory activity in the open field, auditory startle responses and nociception (Scremin et al. 2003). All of the neurological and behavioral effects observed can be affected by cerebral cholinergic transmission or modulation. Since the symptoms experienced by PGW veterans are apparently related to CNS cholinergic functions, we studied, among other responsible factors, alterations in the availability or turnover of ACh and its precursor and degradation product Ch. This study was, thus, designed to determine if exposure of experimental animals to sarin or PB, alone or in combination, in doses and durations that presumably applied to PGW veterans, could elicit delayed or persistent alterations in CNS ACh and Ch levels and in ACh turnover and Ch uptake from blood. Animals were exposed to these AChE inhibitors for three weeks, and the variables mentioned above were analyzed at 2, 4, and 16 weeks after treatment.

Cholinergic neurotransmission plays an important role in memory and learning processes as well as in autonomic regulation, two areas in which manifestations of PGW illness have been found (Haley et al. 2004; Wolfe et al. 1998). Therefore, in this study the sampling of brain

regions for analysis was focused on the area in which cholinergic markers are most prominent (striatum) or the areas that are involved in memory (hippocampus, neocortex, and piriform cortex) or autonomic regulation (mesencephalon, infundibulum).

MATERIALS AND METHODS

Animals: Male Sprague-Dawley (Crl:CD(SD)IGSBR) rats, weighing 250 to 300 g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Laboratories (Kingston, NY). Upon arrival, the animals were quarantined for a week and tested for evidence of disease. They were housed individually in polycarbonate cages in temperature ($21 \pm 2\ ^{o}C$) and humidity ($50 \pm 10\%$) controlled animal quarters maintained on a 12-h light/dark full-spectrum lighting cycle with lights on at 0700 h. Laboratory chow and water were freely available.

Experiments with animal dosing and exposures were conducted at the US Army Medical Research Institute of Chemical Defense (USAMRICD), Aberdeen Proving Ground, MD, and determinations of ACh and Ch levels and turnover were conducted at the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System (VAGLAHS), Los Angeles, CA. The research environment and protocols for animal experimentation were approved at each site by their respective Institutional Animal Care and Use Committees (IACUC). Animal facilities at both institutions are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Materials: Saline (0.9% NaCl) solution, USP, was purchased from Cutter Laboratories, Inc. (Berkeley, CA). Sarin, obtained from US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline before injection. Saline or sarin injection volume was 0.5 ml/kg, s.c. PB was purchased from Sigma-Aldrich (St. Louis, MO) and prepared twice weekly in tap water and provided as drinking water to experimental groups for a 3-week period. Acetone was purchased from Burdick & Jackson (Muskegon, MI), formic acid from J.T. Baker (Phillipsburg, NJ), propionyl chloride from Fluka (Milwaukee, WI), TAPS buffer from Sigma-Aldrich and all other chemicals from Fisher Scientific (Pittsburgh, PA). Thiophenoxide, D4Ch, and ACh and Ch labeled with 9 deuterium atoms (D9ACh and D9Ch, respectively) were synthesized in the Los Angeles' laboratory as previously described (Jenden et al. 1973).

Experimental groups: Separate sets of animals were studied at 2, 4, and 16 weeks after treatment. Within every set, animals were divided into 4 treatment groups. Group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline (0.5 ml/kg s.c.). Group 2 animals received PB in drinking water (80 mg/L) and were injected with saline. PB was provided in drinking water for oral intake to maintain a relatively constant level of inhibition of blood AChE activity throughout the 3-week treatment period (Scremin et al., 2003) (Scremin et al., 2005). Group 3 animals received tap water and were injected with sarin (62.5 ug/kg, s.c., equivalent to 0.5 x LD_{50}). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above. PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 08:00 h. At 09:00 h that Monday morning, injection of either saline (0.5 ml/kg, s.c.) or sarin (62.5 ug/kg, s.c.) was initiated. The injections were given three times (Mondays, Wednesdays, and Fridays at 09:00 h

each day) per week for three weeks. PB in drinking water was terminated and switched to regular tap water at 17:00 h on Friday of the third week. Animal dosing procedures were performed at USAMRICD. After a period of 1, 3, and 15 weeks after treatment, depending on the experimental sets, animals were transported by air-conditioned vans and air-flight to the Laboratory of Neurophysiology at VAGLAHS where they were allowed to recover for a minimum of one additional week before starting assessment of the outcome parameters at 2, 4, and 16 weeks after control, PB, sarin, or PB+sarin treatments. The distribution of animals per group was as follows: 2 weeks post-treatment: control=11, PB=10, sarin=12, sarin+PB=12; 4 weeks post-treatment: control=10, PB=12, sarin=12, sarin+PB=10; and 16 weeks post-treatment: control=8, PB=9, sarin=8, sarin+PB=7.

Observation of signs of intoxication: Animals were observed for signs of cholinergic intoxication for at least one hour following sarin injection. The signs, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination) were scored according to the rating schedule described elsewhere (Shih and Romano 1988).

Determination of levels of brain regional ACh and Ch, Ch uptake from blood and ACh turnover: Animals were anesthetized with halothane (2.5% for induction and 1% for maintenance) in 30% O_2, balance N_2O. A polyethylene (PE50) catheter was inserted in a femoral vein through a cutdown in the inguinal area. After suturing the skin, anesthesia was discontinued and the animals were positioned in a restraining device adapted for introduction into the animal chamber of the microwave fixation apparatus (Gerling Biostat, nominal power = 5kW). Fifteen min after discontinuation of anesthesia, D4Ch (20 μmol/kg of the tosylate salt in saline) was injected intravenously (i.v.), and after a timed interval of 1 min, microwave fixation of the brain was performed immediately following a dose of thiopental (50 mg/kg, i.v.). This served to trace the uptake of D4Ch into the CNS (Ch uptake from blood) and the turnover of D4ACh from D4Ch. The term "turnover" is used here to designate the amount of ACh synthesized from D4Ch during the period between D4Ch injection and microwave fixation of the brain. The rate of synthesis of D4ACh is linear at this time point and it has been shown that this is an effective method to measure the rate of D4ACh synthesis without significantly altering the steady state (Jenden et al. 1974b). The brain was rapidly removed, cooled and dissected into the following brain regions of interest: hippocampus, infundibulum, mesencephalon, neocortex, piriform cortex, and striatum. These tissue sections were homogenized in an ice-cold solution containing 15% 1N formic acid and 85% acetone for analysis of D0ACh, D4ACh, D0Ch, and D4Ch by GCMS. D9ACh and D9Ch were added in precisely known amounts to serve as internal standards for quantitation. The homogenate was centrifuged and the supernatant transferred to clean centrifuge tubes and extracted with diethyl ether. The aqueous residue remaining after the ether extractions was prepared for GCMS determination of Ch and ACh as previously described (Jenden et al. 1973).

Data Analysis: Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data are presented in graphs as means with standard errors of mean (SEM). Main effects for the factors "region" and "treatment" were tested by analysis of variance (ANOVA) (general linear model) followed, if significant (probability for F ratio < 0.05), by multiple contrasts using the Tukey-Kramer multiple comparisons test. A probability level of <0.05 was used to declare differences as statistically significant. The

interaction between the factors "region" and "treatment" was also studied, the implication being that if this was significant, then it could be assumed that the treatment effects were dependent on the brain region studied. A final analysis of treatment effects was performed by analysis of covariance (ANCOVA) using the region's values for each variable as covariates.

RESULTS

No sign of cholinergic toxicity was found in animals during the three weeks of treatment with PB, sarin or the combination of both AChE inhibitors, which was in line with our previous observation in this experimental model (Scremin et al. 2003; Scremin et al. 2005).

Regional differences: ANOVA indicated high significance for the factor brain regions ($P<10^{-6}$) but with no interaction between brain regions and treatments ($P = 0.87$) or brain regions and weeks post-treatment ($P = 0.63$). Thus, the region's values for each variable averaged over weeks and treatments, shown in Table 1, were used as covariates to control for this confounding factor when testing differences among treatments. Levels of D4ACh in infundibulum were below the limit of accurate detection.

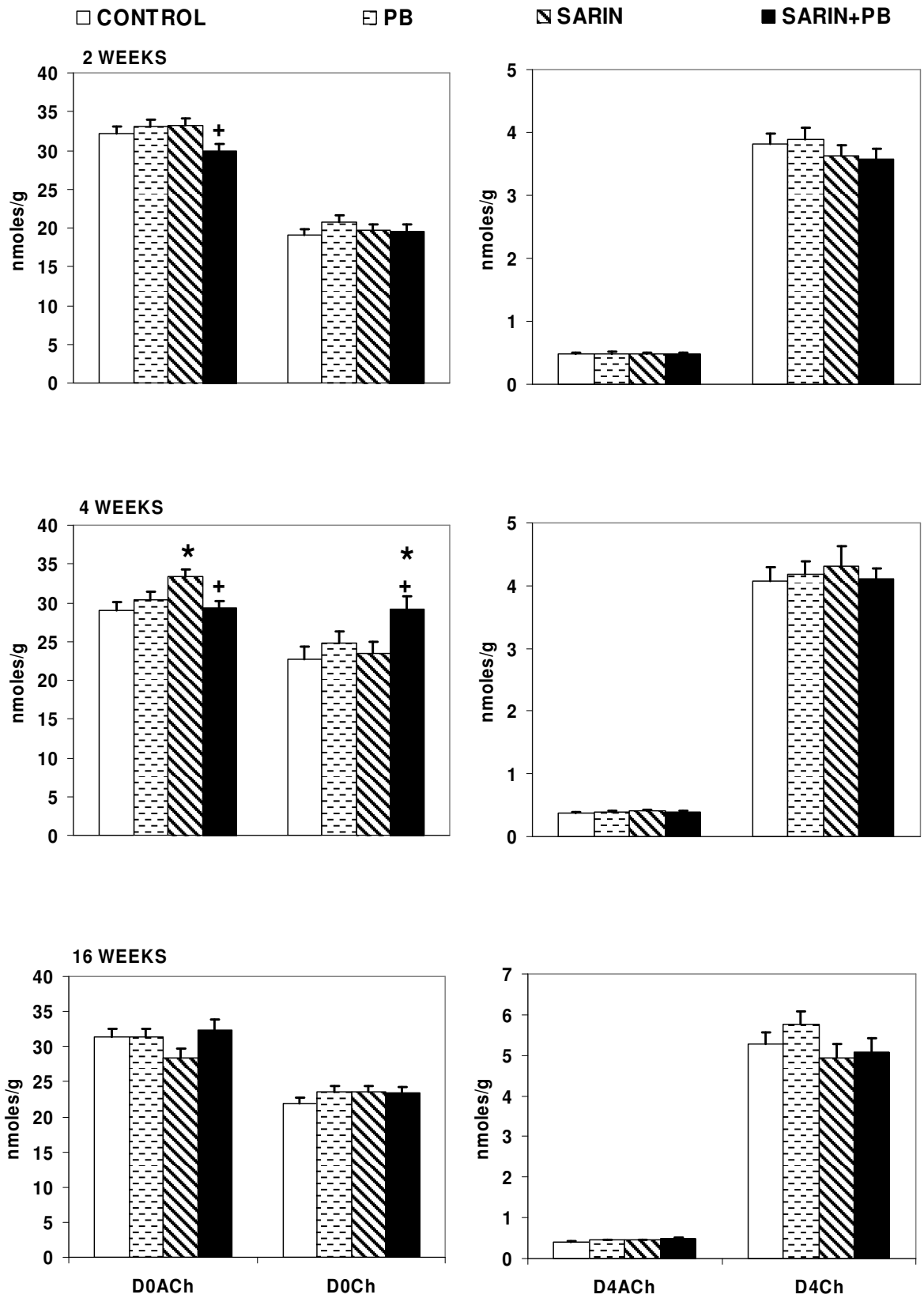
Treatment differences: The means and S.E. of every variable for all treatments and weeks post-treatment, adjusted by analysis of covariance as described above, are shown in Figure 1. ANCOVA indicated statistical significance for the factor treatment for D0ACh at two weeks post-treatment ($P = 0.032$) and four weeks post-treatment ($P = 0.006$), and for D0Ch at four weeks post-treatment ($P = 0.016$). Multiple comparisons among means in those cases were performed (Tukey-Kramer test, $P<0.05$), and the results indicated that D0ACh was lower in the sarin+PB group than in the sarin group at two and four weeks, while the sarin group was higher than the control group at four weeks (Fig.1). At four weeks post-treatment, D0Ch was significantly higher in the sarin+PB group than in the control group (Fig. 1). No statistical significance among treatment groups was found at sixteen weeks post-treatment for any of the variables.

Table 1: Mean ± S.E. (nmoles/g) across treatments and weeks post-treatment of all variables studied in every brain region. ANOVA indicated high significance for the factor brain regions but with no interaction between brain regions and treatments or brain regions and weeks post-treatment. Thus, the region's values for each variable were averaged over weeks and treatments. Data were collected from 121 animals.

Brain Region	D0ACh	Diff. from*	D0Ch	Diff. from*	D4ACh	Diff. from*	D4Ch	Diff. from*
1.Hippocampus	25.4 ± 0.55	2,3,4,5,6	19.4 ± 0.69	2,6	0.35 ± 0.013	3,4,6	2.07 ± 0.063	2,3,4,5
2.Infundibulum	18.0 ± 0.74	1,3,5,6	31.5 ± 0.86	1,3,4,5,6	---	---	10.79 ± 0.379	1,3,4,5,6
3.Mesencephalon	30.4 ± 0.61	1,2,4,5,6	20.1 ± 0.65	2	0.21 ± 0.011	1,4,5,6	3.00 ± 0.094	1,2,5,6
4.Neocortex	17.0 ± 0.44	1,3,5,6	19.4 ± 1.35	2,6	0.44 ± 0.017	1.3.6	3.60 ± 0.109	1,2,5,6
5.Piriform Ctx	34.3 ± 0.79	1,2,3,4,6	22.7 ± 0.69	2	0.41 ± 0.014	3,6	5.51 ± 0.165	1,2,3,4,6
6.Striatum	60.2 ± 1.16	1,2,3,4,5	23.6 ± 0.79	1,2,4	0.77 ± 0.019	1,3,4,5	1.76 ± 0.063	2,3,4,5
Total	31.2 ± 0.60		22.7 ± 0.38		0.33 ± 0.020		4.33 ± 0.127	

* Different from regions listed by Tukey-Kramer multiple pairwise comparisons test, $P<0.05$.
--- Levels of D4ACh in infundibulum were below the limit of accurate detection.

Figure 1 (next page): D0ACh, D0Ch, D4ACh, and D4Ch tissue concentrations (nmoles/g) for all treatment conditions at 2, 4 and 16 weeks post-treatment. Bars represent treatment group means and brackets S.E. of all regions in all animas, adjusted by analysis of covariance. Statistical significance for the factor treatment in ANCOVA was reached for D0ACh at 2 weeks post-treatment ($P =0.032$) and 4 weeks post-treatment ($P = 0.006$), and for D0Ch at 4 weeks post-treatment ($P = 0.016$). Multiple comparisons among means in those cases were performed (Tukey-Kramer test, $P<0.05$) and the results are indicated as (*) = significantly different from control group, and ($^{+}$) = significantly different from sarin group.


CONTROL
PB
SARIN
SARIN+PB
2 WEEKS
4 WEEKS
16 WEEKS
nmoles/g
D0ACh
D0Ch
D4ACh
D4Ch

DISCUSSION

This study was intended to determine whether exposure of experimental animals to sarin or PB, alone or in combination, in doses and durations that presumably applicable to PGW veterans, could elicit delayed or persistent alterations in CNS ACh and Ch levels and in ACh and Ch turnover. However, the model we used here was the worst case scenario for the PGW exposure in which service members did not report any symptom of miosis, an initial sign of aerosol exposure. In our previous use of this model, with the same doses of AChE inhibitors used in the present report, PB decreased red blood cell AChE activity to about 51%, sarin to 33% and the combination of PB and sarin to 27% of baseline during the drug administration period (Scremin et al. 2005) . Although brain AChE was not measured during drug administration, previous data indicated that at the levels of blood AChE observed, significant inhibition of brain AChE activity could be reasonably assumed (Roberson et al. 2001; Shih 1983; Shih et al. 1990). At three weeks post-treatment the inhibition was reduced considerably in magnitude for sarin and sarin+PB groups, while it had recovered to normal levels in the case of PB treatment. Our model of low-level sarin and PB treatment regimen had indicated delayed effects on exploratory activity in the open field, auditory startle responses and nociception (Scremin et al. 2003). Presumably, all of these phenomena can be affected by cerebral cholinergic transmission or modulation.

In the current study, the values of unlabeled (i.e., endogenous D0ACh and D0Ch) and labeled (i.e., exogenous D4ACh and D4Ch) ACh and Ch observed are in general agreement with those reported previously with the presently used methodology and under similar experimental conditions for neocortex, hippocampus and striatum (Freeman et al. 1979; Jenden et al. 1974a; Jenden et al. 1976; Jope 1979; Jope and Jenden 1979; Russell et al. 1981). No previous data are available, however, with this methodology regarding piriform cortex and infundibulum.

The general pattern of brain regional variations of the variables under study was not affected by the length of time post-treatment in control animals, indicating a lack of age effects over the time span of the study. The levels of D0Ch and D4Ch were highest in the infundibulum. The cause of this phenomenon can be found in the behavior of the blood-brain barrier, which normally restricts the movement of Ch through brain capillaries and is known to be absent in this region (Davson et al. 1987). Thus, levels of D4Ch that reflect Ch uptake from blood and of D0Ch that represents Ch abundance in the region exceed those of all other brain regions in which the uptake of this base from blood depends on the activity of Ch transporters rather than on free diffusion as in the infundibulum. The lack of an effect of treatments on D4Ch indicates that the low levels of AChE inhibitors under study did not elicit changes in the blood-brain barrier under the present experimental conditions.

It is true that uptake of Ch by the high affinity Ch uptake system into the ACh synthesis compartment correlates under many experimental paradigms with ACh turnover. However, most of the Ch uptake measured with the current method through D4Ch may be via low affinity systems that will not necessarily correlate with the ACh synthesis process. This may explain why no simple relationships between D4ACh and D4Ch are found in the present data.

The endogenous Ch (D0Ch) concentration has been found to be increased in conditions of tissue injury, decreased cerebral blood flow and hypoxia (Jenden 1991; Scremin and Jenden 1996; Scremin and Jenden 1993; Scremin and Jenden 1992; Scremin and Jenden 1989) as well as after prolonged repeated treatment with sublethal doses of the nerve agent soman (Shih et al. 1990) and diisopropylfluorophosphate (DFP) (Russell et al. 1981). The fact that PB and sarin

treatments did not affect the levels of D0Ch beyond 4 weeks post-treatment argues against tissue damage in this experimental model.

Treatments induced few changes, limited only to increased D0ACh and D0Ch of animals treated with sarin or sarin+PB at 2 and 4 weeks post-treatment. No statistically significant change of any variable was found 16 weeks post-treatment. The lack of persistent or delayed effects on cholinergic neurochemistry by the treatment regimens used in the present model of low-level AChE inhibitor exposure is in line with our previous study of brain choline-acetyltransferase activity and muscarinic receptor binding. The previous study did not show any significant change either in cholinergic neurochemistry, except for down-regulation of muscarinic receptors 2 weeks post-treatment (Scremin et al. 2003).

We are not aware of any studies addressing the effects of low-level, non-symptomatic OP AChE inhibitors or PB on central ACh and Ch availability or turnover. A number of studies have reported changes in these variables after exposure to levels that produce toxic symptoms of OP AChE inhibitors. Following a single high dose of soman (0.9 x LD50), brain ACh content increases reaching a peak in cerebral cortex and striatum at 2 h and in hippocampus and midbrain at 3 h (Shih 1982). The change persists for 2 h in the midbrain and striatum, 8 h in the cerebral cortex and 16 h in the hippocampus. Increase in brain ACh levels has also been reported with DFP (Russell et al. 1981) and paraoxon (Wecker and Dettbarn 1979). Ch levels are enhanced after a soman challenge in all areas of the brain except the striatum, reaching a maximum between 10 and 40 min, and returning to control levels in approximately 3 h (Shih 1982). Repeated administration of three times per week of sublethal, but symptomatic doses of soman induced enhancement of tissue Ch levels after 6 weeks, but no changes in tissue ACh (Shih et al. 1990).

The reported effects of OP AChE inhibitors on ACh turnover are mixed. Chlorpyrifos depressed and parathion enhanced ACh synthesis on acute administration (Karanth and Pope 2003), soman decreased ACh turnover in hippocampus and cerebral cortex (Jenden and Russell 1991), while no effects were found with DFP (Russell et al. 1981). Regarding Ch uptake from blood estimated by the ratio of D4Ch/(D0Ch + D4Ch), no effects of soman, physostigmine or their association were reported (Jenden and Russell 1991). Russell et al. also reported lack of effects of DFP on whole brain D4Ch uptake (Russell et al. 1981).

In conclusion, this study attempted to detect possible persistent or delayed effects of low-level exposure to the AChE inhibitors sarin and PB, alone or in combination, on the endogenous levels of ACh and Ch, turnover of ACh or Ch uptake from blood. Only small effects on ACh and Ch availability were detected 2 and 4 weeks post-treatment, and none at 16 weeks post-treatment. Thus, the results from these experiments do not support a delayed or persistent alteration in cholinergic function after exposure to low doses of PB and/or sarin.

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ABBREVIATIONS

AChE=acetylcholinesterase
CNS=central nervous system
D0ACh=endogenous acetylcholine
D0Ch=endogenous choline
D4ACh=acetylcholine labeled with 4 deuterium atoms
D9ACh=acetylcholine labeled with 9 deuterium atoms
D4Ch=choline labeled with 4 deuterium atoms
D9Ch=choline labeled with 9 deuterium atoms
DFP=diisopropylfluorophosphate
GCMS=gas-chromatograph mass-spectrometry
OP=organophosphorus compound
PB=pyridostigmine bromide
PGW=Persian Gulf War.